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Listeriolysin O-Mediated Calcium Influx Potentiates Entry of *Listeria monocytogenes* into the Human Hep-2 Epithelial Cell Line

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To investigate factors which modulate the entry of *Listeria monocytogenes* into mammalian cells, we have analyzed the role of Ca^{2+}. We show that *L. monocytogenes* induced Ca^{2+} transients into the human Hep-2 epithelial cell line. The nonpathogenic species *L. innocua* or a *L. monocytogenes* mutant strain defective in listeriolysin O (LLO) production was unable to induce these calcium fluxes. Addition of plasma membrane calcium channel antagonists or chelation of extracellular calcium markedly reduced *L. monocytogenes* entry. In contrast, chelation of host cytosolic Ca^{2+} or blockade of Ca^{2+} release from intracellular stores did not affect invasion. These results indicate that *L. monocytogenes*-induced mobilization of extracellular Ca^{2+} by LLO and activation of downstream Ca^{2+}-dependent signaling are required for efficient cell invasion.

Listeria monocytogenes has become a model system for the molecular study of host-pathogen interactions. Much attention has focused in the recent years on two aspects of its intracellular lifestyle, i.e., how normal cellular receptors and downstream signaling mechanisms are hijacked by the bacterium to promote its entry into nonprofessional phagocytic cells and how actin polymerization inside the cytosol is used by the bacterium to spread from cell to cell and to stay in an intracellular compartment, thus escaping some specific host defenses (7, 29).

The ability of *L. monocytogenes* to invade various host cells is an important determinant for pathogenesis (8). Entry of *L. monocytogenes* into epithelial cells, which is reminiscent of the classical model of zipper phagocytosis, occurs through the interaction of two bacterial surface proteins with their cellular receptors. InlA (internalin) promotes phagocytosis in cell lines expressing its receptor, the adhesion protein E-cadherin, whereas InlB interacts with several cellular ligands: two protein receptors, c-Met (the hepatocyte growth factor-scattering factor receptor) and gC1qR, but also with glycosaminoglycans (3, 14, 19, 24). It activates a number of signaling pathways, including phosphatidylinositol (PI) 3-kinase and NF-κB (13, 17). Signaling events elicited by InlA and InlB lead to actin-mediated zippering of the host membrane around the bacterium and internalization (9).

Ca^{2+} is very important in a great variety of eucaryotic cell signaling processes (exocytosis, contraction, metabolism, gene transcription, fertilization, and proliferation) and is an important regulator of actin microfilaments (for a review, see reference 1). In this work, we have analyzed the role of Ca^{2+} in the entry of *L. monocytogenes* strain EGD into the human Hep-2 epithelial cells. In these cells, entry of *L. monocytogenes* is InlB dependent (5).

We first measured the levels of intracellular Ca^{2+} ($[Ca^{2+}]_i$) in cultured Hep-2 cells with the fluorescent probe Indo 1-AM after challenge with wild-type *L. monocytogenes*. A total of 5 to 20 cells per field were chosen for individual Ca^{2+} analysis. As shown in Fig. 1A (left panel), addition of wild-type *L. monocytogenes* strain EGD resulted in a rapid and transient increase in $[Ca^{2+}]_i$ which lasted from 10 to 20 min after bacterial contact and then returned to basal levels. Synchronization of the entry process (prior to $[Ca^{2+}]_i$ measurement) by centrifuging the bacteria on the cells at 4°C to allow bacterial attachment but not invasion gave a stronger and sharper $[Ca^{2+}]_i$ increase (middle panel) very similar to the $[Ca^{2+}]_i$ transients evoked by the calcium ionophore A23187I (right panel). By comparison with the increase obtained with the calcium ionophore A23187I (10 μM), which is known to increase the calcium concentration from 0.1 μM to approximately 1 μM, the $[Ca^{2+}]_i$ increase induced by *L. monocytogenes* in the conditions used was twofold lower. Blocking the entry process but not adherence with cytochalasin D, a toxin that blocks actin rearrangements, did not affect the *L. monocytogenes*-induced $[Ca^{2+}]_i$ increase (Fig. 1B, left panel), demonstrating that this signal occurs early in the infectious process at the adherence step. Indeed, an *inlB* mutant strain which is markedly affected in its ability to enter Hep-2 cells, although fully capable of attaching to these cells, still induced $[Ca^{2+}]_i$ increase (middle panel), while *L. innocua*, a nonpathogenic, noninvasive, and nonadherent *Listeria* species, did not trigger $[Ca^{2+}]_i$ fluxes (right panel).

Several reports have highlighted that listeriolysin O (LLO), a member of the pore-forming cholesterol-dependent cytolysins, is a potent signaling molecule which can activate phosphoinositide metabolism and MAPK and NF-κB transduction pathways (15, 25, 26, 27). Binding of LLO to cholesterol-containing membranes is followed by insertion, oligomerization, and formation of a pore 20 to 30 nm in diameter that may allow the passage of small ions such as Ca^{2+}. In addition, LLO is not produced by *L. innocua* strains (12). Thus, we questioned the role of LLO in $[Ca^{2+}]_i$ increases. LLO is a secreted protein, but it is also found associated to the cell surface (2, 21). In all of these experiments, bacteria were washed twice in phosphate-buffered saline (PBS) to remove any trace of LLO

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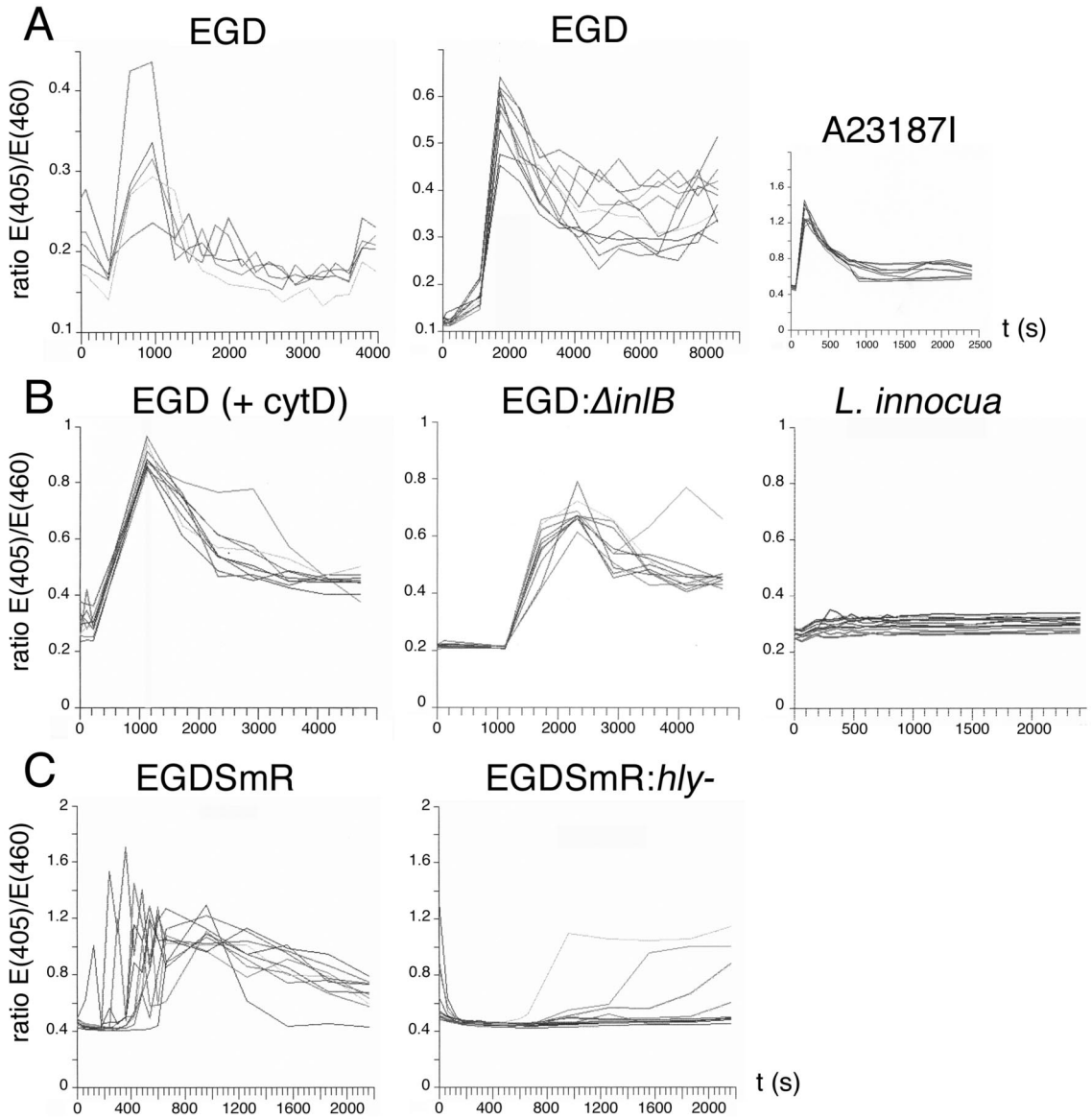

FIG. 1. *L. monocytogenes* strain EGD induces $[Ca^{2+}]_i$ increases in epithelial Hep-2 cells. Hep-2 cells were grown on Lab-Tek chambered coverglass (Nunc) 2 days prior to the experiment. Cells were loaded with 3 μM Indo 1-AM and pluronic acid at a 0.01% final concentration at 37°C for 30 min. Loading and calcium experiments were carried out in serum-free DMEM (Gibco; 1.8 mM Ca^{2+}). Cells were stimulated by *L. monocytogenes* or *L. innocua* at a multiplicity of infection of 50 to 100. A23187I was added at a final concentration of 10 μM. Bacteria were washed twice in PBS and then centrifuged on cells at 200 × *g* for 1 min, and Ca^{2+} measurements were performed at 23°C with an ACAS-Meridian imaging system. Values along the horizontal axis represent time in seconds, and values along the vertical axis represent the amplitude of the Ca^{2+} response in arbitrary units. For details of the varions panels, see the text.

present in the culture supernatant because of toxicity on the host cell.

As shown in Fig. 1C, comparison of the wild-type and the nonhemolytic isogenic strains showed that wild-type *L. monocytogenes* strain EGDSmR triggers a $[Ca^{2+}]_i$ increase while its nonhemolytic counterpart EGDSmR (lacking *hly*) does not. With the mutant strain lacking *hly*, interestingly, approximately 15% of Hep-2 cells gave a delayed and smaller Ca^{2+} response. Wadsworth and Goldfine had reported changes in host cell $[Ca^{2+}]$ during phagocytosis of *L. monocytogenes* by J774 macrophage-like cells. Using mutants deficient in one or more virulence factors, including LLO and PI-PLC and PC-PLC, the two other phospholipases of *L. monocytogenes*, those authors showed that these exotoxins were involved in the three sequential $[Ca^{2+}]$ waves induced during *L. monocytogenes* uptake by J774 cells (30). Based on these results, our observations with the nonhemolytic mutant lacking *hly* can be explained by a minor role of PI-PLC and PC-PLC in the Ca^{2+} response in Hep-2 cells.

Using the gentamicin survival assay as previously described (10) for investigation of whether a $[Ca^{2+}]_i$ increase was involved in bacterial entry, we tested the invasion capacity of the

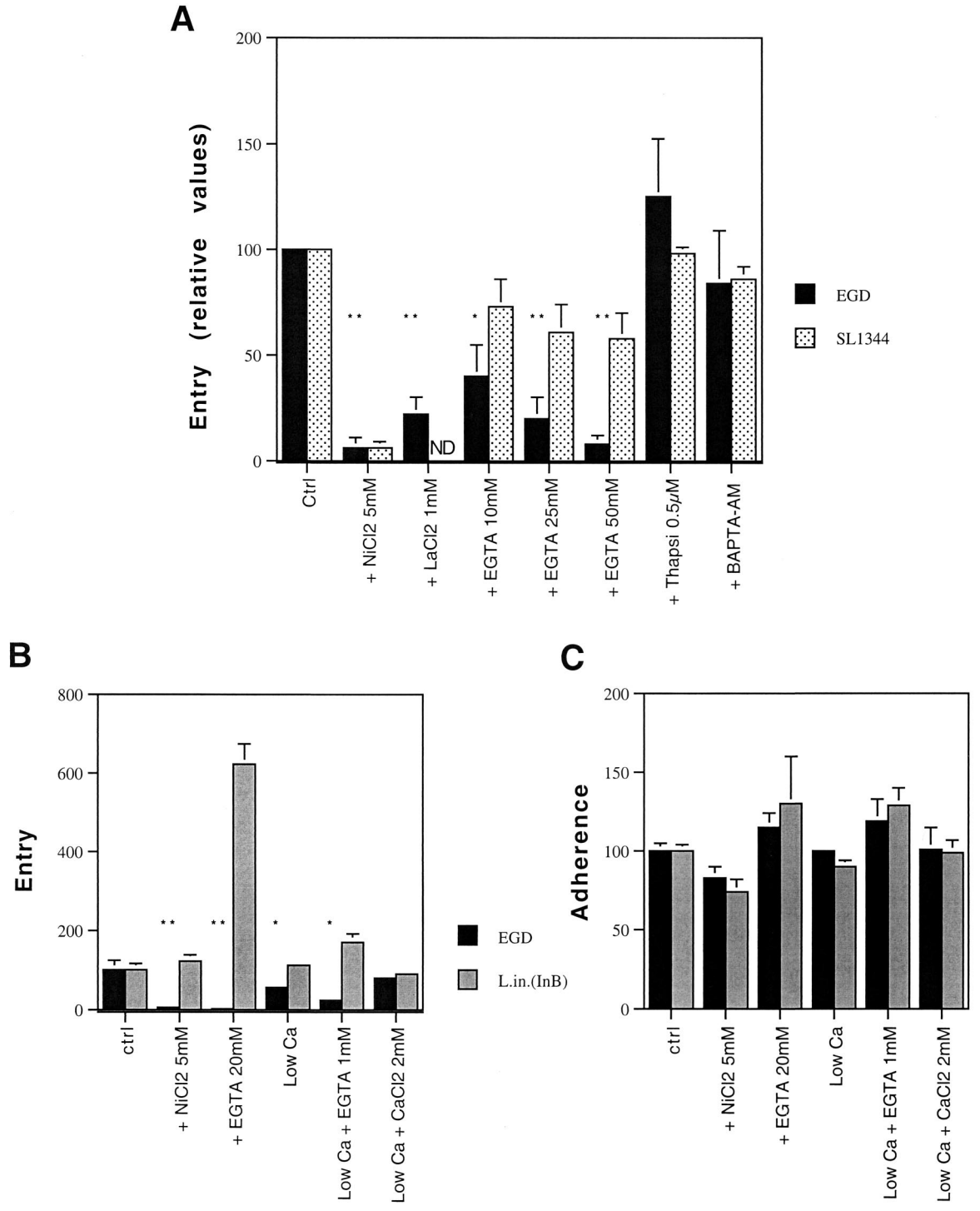

FIG. 2. Extracellular Ca^{2+} is required for efficient entry of *L. monocytogenes* into Hep-2 cells. Ctrl, control. (A) Exponentially growing bacteria were added to the cells cultivated in 24-well plates 2 days prior to the experiment at a multiplicity of infection of 10 and 100 for *Salmonella* and *Listeria*, respectively. After 1 h of infection in DMEM, the cells were washed several times and DMEM-containing gentamicin (10 μg/ml) was added for 1 h to kill the extracellular bacteria. Hep-2 cells were washed and lysed in PBS containing Triton (0.2%), and lysates were plated on brain heart infusion plates for bacterial counting. Entry of *L. monocytogenes* strain EGD and *S. enterica* serovar Typhimurium strain SL1344 (kindly given by M.-Y. Popoff) was normalized to 100. All of the inhibitors were added 15 min prior to the infection and kept during the 1-h infection period. The viability and the number of the cells were constantly monitored during the experiment. Results are expressed as means ± standard deviations of three to five independent experiments performed in duplicate. For *S. enterica* serovar Typhimurium, the graph represents the means ± standard deviations of one experiment performed in duplicate. (B) Entry of *L. monocytogenes* strain EGD and *L. innocua* (InlB-SPA) was performed in the same conditions as described for panel A. The low Ca^{2+} DMEM was from Gibco (BRL). (C) For adhesion assays, infection was performed with *L. monocytogenes* strain EGD in the same conditions as described for panel A. After the 1-h infection period, the cells were washed several times to remove nonadherent bacteria and then the cells were lysed and lysates were plated for counting. In this type of assay, adherent bacteria include the few bacteria that have been internalized. Statistical analysis (Student's *t* test) was applied to the results obtained for all panels. ** indicates $P < 0.005$, which is considered very significant compared to the control data (medium only); * indicates $P < 0.05$, which is considered significant.

nonhemolytic mutant compared to that of the isogenic wild-type strain for entry into Hep-2. We found that the percentages of entry for 1 h of infection and 30 min of gentamicin treatment at a multiplicity of infection of 50 were 14 ± 1.5% for wild-type EGD SmR versus 3.7 ± 0.7% for EGD SmR lacking *hly*. These results suggested that $[Ca^{2+}]_i$ increases may play a role in the entry process.

To more specifically address the role of Ca^{2+} in bacterial entry, a pharmacological approach was used as previously described for *Salmonella enterica* serovar Typhimurium (20). $[Ca^{2+}]_i$ increases originate either from the external medium through opening of plasma membrane calcium channels or from release from internal stores through activation of inositol phosphate 3 receptor. We tested the effect on *L. monocytogenes* invasion of Hep-2 cells of the nonspecific calcium channel antagonists nickel chloride or lanthanum chloride ($NiCl_2$ or $LaCl_2$) or chelation of external calcium with EGTA to block Ca^{2+} influx from the external medium. We also tested the effect of internal calcium chelation with BAPTA-AM (1,2-bis[2-amino-phenoxy]ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis [acetoxymethyl] ester) (10 μM) and the effect of emptying the main $[Ca^{2+}]_i$ store from the endoplasmic reticulum with thapsigargin (0.5 μM) on *L. monocytogenes* invasion of Hep-2 cells. In all of these experiments with inhibitors, we also assessed the entry *of S. enterica* serovar Typhimurium into Hep-2 cells as a control.

As shown in Fig. 2A, removal of calcium from the assay buffer with EGTA significantly reduced bacterial entry in a dose-dependent manner. Consistent with this result, addition of nickel or lanthanum chloride prevented the internalization of *L. monocytogenes*. In the same experimental conditions, the entry rate of the noninvasive Δ*inlB* mutant was 7.5 ± 2.5%. In contrast, chelation of intracytosolic calcium with BAPTA-AM or emptying the $[Ca^{2+}]_i$ stores and preventing refilling with thapsigargin did not affect bacterial uptake. As previously shown, entry of *S. enterica* serovar Typhimurium into Hep-2 cells was also dependent on extracellular $[Ca^{2+}]_i$ influx. Taken together, these results indicate that $[Ca^{2+}]_i$ influx from the external medium is required for efficient *L. monocytogenes*-induced bacterial uptake.

To determine whether Ca^{2+} signaling can play a role in InlB-mediated entry in the absence of LLO, we made use of *L. innocua* expressing the LRR domain of InlB (InlB-SPA), a region shown to be sufficient to promote entry (4). Entry of *L. innocua* (InlB-SPA) and that of *L. monocytogenes* strain EGD were analyzed in the presence of nickel chloride ($NiCl_2$), a nonspecific calcium channel antagonist, or in the presence of the external calcium chelator EGTA. A low-calcium cell culture medium (0.1 μM Ca^{2+}) was also tested and compared to the normal Dulbecco's modified Eagle's medium (DMEM) (1.8 mM Ca^{2+}). As shown in Fig. 2B, addition of $NiCl_2$ or EGTA blocked entry of *L. monocytogenes* strain EGD but not that of *L. innocua* (InlB-SPA). Since *L. innocua* (InlB-SPA) does not produce LLO, these results suggest that the Ca^{2+}-positive effect on invasion is linked to the presence of LLO. Intriguingly, entry of *L. innocua* (InlB-SPA) is more efficient in the absence of external Ca^{2+} ions. How this relates to the presence of Ca^{2+} binding sites on the N-terminal cap domain of InlB (18) is under current investigation.

Adherence of *L. monocytogenes* strain EGD and *L. innocua* (InlB-SPA) was not significantly altered by the presence of $NiCl_2$ or EGTA in the culture medium (Fig. 2C). These results suggested that Ca^{2+} increase is mostly required for the internalization process of wild-type *L. monocytogenes*. Interestingly, addition of the calcium ionophore A29187I did not trigger entry of the nonpathogenic *L. innocua* into Hep-2 cells (data not shown) nor did it increase the entry rate of *L. monocytogenes*, suggesting that a generalized Ca^{2+} increase does not promote entry and that a localized Ca^{2+} increase at the contact between bacteria and the host membrane is necessary to promote bacterial uptake.

In conclusion, Ca^{2+} appears to be a critical factor for entry of *L. monocytogenes* into Hep-2 cells. A recent report shows that LLO forms Ca^{2+}-permeable pores, leading to $[Ca^{2+}]_i$ oscillations in the epithelial HEK 293 cell line (22). In agreement with these results, we showed that the Ca^{2+} response observed in Hep-2 cells is due to LLO. We also showed that Ca^{2+} changes induced by *L. monocytogenes* modulate the internalization process and can probably control another function(s). The role of Ca^{2+} in phagocytosis has been largely studied but is still controversial. It is thought to act in many different ways through actin-binding proteins, protein kinase C PKC, and vesicle exocytosis as a source of membrane during phagocytosis. LLO is thus a multifunctional protein that not only mediates escape of the bacterium from the primary and secondary phagosomes but also activates various signaling pathways inside the host cell (i.e., MAPK and NF-κB pathways) which result in the expression of specific adhesion molecules and chemokines (15, 16, 23, 28) and, as shown here, are involved in calcium signaling and entry. Interestingly, the cellular receptor for LLO is cholesterol, a molecule highly enriched in lipid rafts which are considered important signaling domains (6, 11). It is now important to investigate how Ca^{2+}, LLO, and rafts are interconnected and control entry into nonphagocytic cell lines.

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